

# CHEMICAL MODIFICATION OF COLLAGEN BY THE MANNICH REACTION\*

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## ABSTRACT

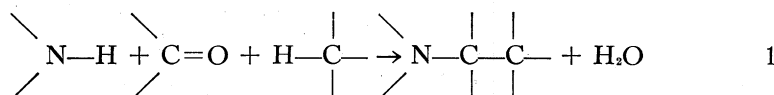
A variety of aliphatic compounds containing active hydrogen atoms have been tested in Mannich or Mannich-type reactions with collagen for tanning properties and the products thus obtained have been tested as new substrates for subsequent mineral retannages. None of the compounds tested had any tanning properties which were improvements over simple formaldehyde tannages under the conditions used in this investigation; only one compound, malonic acid, gave a new substrate which had enhanced binding power for chrome.



## INTRODUCTION

A variety of reactions has been used to modify the chemical structures of proteins; however, if the protein is to retain its physical integrity during the reaction, mild conditions must prevail. A reaction that is particularly suited, although seldom used, is the Mannich reaction (1). This reaction, which can be carried out under a variety of conditions, has even been proposed (2) as a possible step in the biosynthesis of some natural products. Fraenkel-Conrat (3), in his studies on the reaction of formaldehyde with proteins, pointed out the possibility of a Mannich-type reaction between the amino groups of the protein, formaldehyde, and the amido or guanidino groups also of the protein. Windus also postulates a Mannich reaction in his tannages involving resorcinol and formaldehyde (4) as well as melamine and formaldehyde (5).

The Mannich reaction involves the condensation of an amine, a carbonyl compound, and a compound with an "active" hydrogen atom and can be formulated in the broadest of terms as follows:



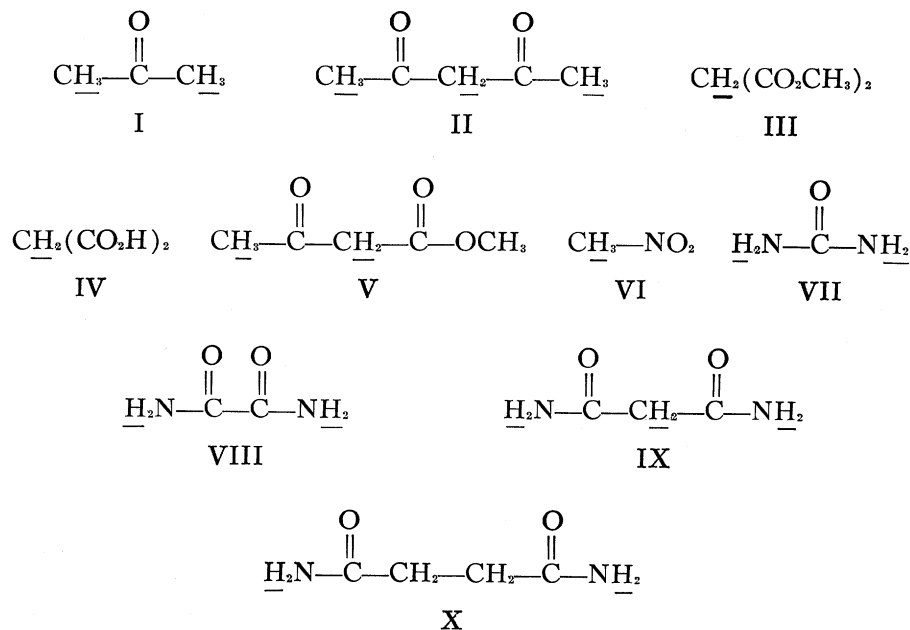
The amine component can be a primary or secondary amine or ammonia; the carbonyl compound most generally used is formaldehyde; and the "active" hy-

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hydrogen-atom-containing compound can be any compound with a strong electron withdrawing group or groups attached to the same carbon atom as the "active" hydrogen atom (neglecting aromatic compounds; such as, phenols). Electron withdrawing groups include nitro, keto, carboxyl, carboalkoxy, and amido groups. Keto groups and nitro groups are strong enough that a single group supplies sufficient activation; however, more than one of the other groups is necessary.

In the application of this reaction to proteins, the  $\epsilon$ -amino groups of lysine and hydroxylysine residues as well as the imidazole groups of histidine residues serve as the amine component; formaldehyde serves as the carbonyl compound; and a variety of "active" hydrogen-atom-containing compounds can be used. Applied to collagen, in the form of animal hides or skins, this reaction might give a stable tannage or serve as a pretreatment of hides and skins for subsequent mineral tannages. Therefore, a study was begun to survey a variety of "active" hydrogen-atom-containing compounds to test the above possibilities. The compounds surveyed were acetone (I), acetylacetone (II), dimethyl malonate (III), malonic acid (IV), methyl acetoacetate (V), nitromethane (VI), urea (VII), oxamide (VIII), malonamide (IX), and succinic diamide (X). The diamides have been included because the amido hydrogen atoms are "active" enough to take part in a Mannich type reaction (3, 6). The "active" hydrogen atoms are underlined.



This paper reports the results of this survey.

## EXPERIMENTAL

Percentages are based on the drained, pickled weight (DPW) of the sheepskins. Small-scale treatments and tannages were carried out in glass bottles on a tumbling machine. The formaldehyde was used in the form of a 37 percent solution in water (formalin). The percentages reported are for anhydrous formaldehyde, not for the formalin. The reagents, which were reagent grade or better, were used without further purification.

**Mannich Treatment at pH 2.**—Pieces of pickled cabretta skins were placed in a 100 percent float of a four percent sodium chloride solution, and the "active" hydrogen-atom-containing compound was added in the amount of ten percent of the DPW of the skin. The pH was adjusted with a ten percent sulfuric acid solution to about 2, and the samples were tumbled for one hour. Formaldehyde was added in the amount of ten percent of the DPW of the skin, and tumbling was continued overnight. The pH was adjusted to about 4.5 with a saturated sodium bicarbonate solution, and the samples were tumbled for two more hours. They were washed for at least one hour in running water, dried at ambient room conditions, and staked. The shrinkage temperature ( $T_s$ ) (7) was measured on the wet samples immediately after washing.

**Mannich Treatment at pH 4.**—Pieces of pickled cabretta skins were placed in a 200 percent float of a five percent sodium chloride-five percent sodium formate solution and were tumbled for 30 minutes. Formaldehyde was added in the amount of five percent of the DPW, and tumbling was continued for six hours. The "active" hydrogen-atom-containing compound was added in the amount of five percent of the DPW, and the tumbling was continued overnight. The skins were washed, dried, and staked as above. The  $T_s$  was measured before drying.

**Mannich Treatment at pH 8.**—Pieces of pickled cabretta skins were placed in a 200 percent float of a five percent sodium sulfate-seven percent sodium bicarbonate solution and were tumbled for 30 minutes. Formaldehyde and the "active" hydrogen-atom-containing compound were both added in the amount of five percent of the DPW in the same sequence as in the treatment at pH 4. Tumbling was continued overnight, and the skins were then washed, dried, and staked as above. The  $T_s$  was measured before drying.

**Mannich Treatment at pH 10.**—Pieces of pickled cabretta skins were equilibrated in a 100 percent float of a 2 *N* sodium carbonate solution for three hours. This procedure was adapted from one recommended by Seligsberger and Sadlier (8) for tanning with formaldehyde. Formaldehyde was added in the amount of five percent of the DPW, and the samples were tumbled for one hour. The "active" hydrogen-atom-containing compound was added in the amount of five percent of the DPW, and tumbling was continued under a heat lamp

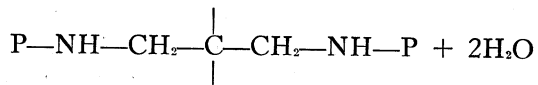
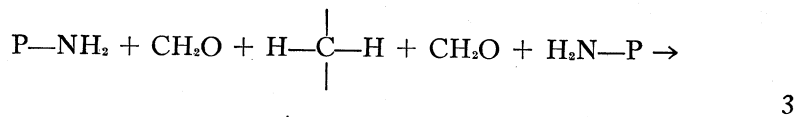
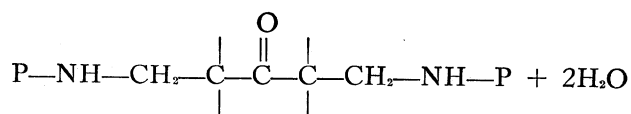
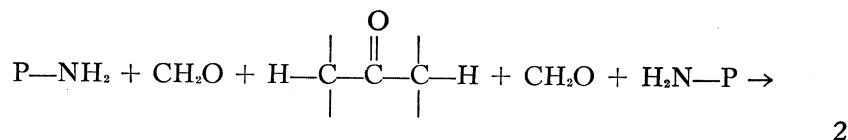
for three hours. The temperature reached approx. 55°C. The skins were then wrung to remove the excess liquid and were allowed to dry overnight at ambient room conditions. The dried samples were placed in a 200 percent float of a four percent sodium chloride solution, and the pH was adjusted to about 5.5 with a ten percent sulfuric acid solution. They were tumbled in this solution for two hours, washed, dried, and staked as above. The  $T_s$  was measured before final drying.

**Chrome Tanning.**—Samples from the various treatments were washed and placed in a 100 percent float of a four percent sodium chloride solution. The pH was adjusted to about 2.5 with a ten percent sulfuric acid solution; and, when it stabilized, they were tumbled for 30 minutes. Tanolin R‡ was added in the amount of nine percent of the DPW in the form of either an 18 or a 36 percent solution in three portions at 20 minute intervals. The samples were tumbled overnight. The pH was adjusted slowly to about 4 with a four percent sodium bicarbonate solution. When the pH remained stable, the skins were tumbled for two hours, washed, dried, and staked. The  $T_s$  was measured on the wet samples immediately after washing.

The  $Cr_2O_3$  contents of the chrome-tanned products were determined by the alkaline fusion method using a sodium thiosulfate titration, as prescribed by Official Methods of Analysis of the ALCA (9).

## RESULTS AND DISCUSSION

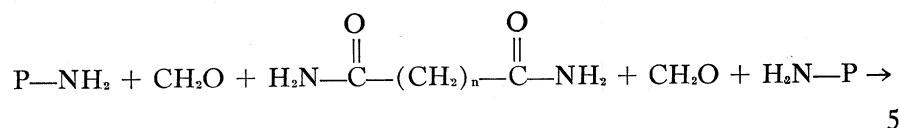
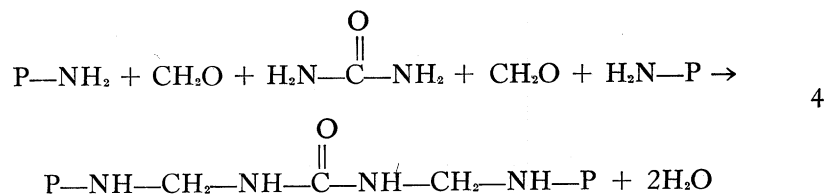
All of the compounds (I through X above) surveyed in this study are polyfunctional with respect to "active" hydrogen atoms and could, with formaldehyde, act as crosslinking agents as exemplified by equation 2.



P = protein

‡Mention of brand or firm names does not constitute an endorsement by the Department of Agriculture over others of a similar nature not mentioned.

This is a simplified equation; and, since these compounds all contain more than two "active" hydrogen atoms, much more complex reactions could and probably do occur. In fact, crosslinking by the Mannich reaction is possible even if the active hydrogen atoms are on the same carbon atom, provided there are at least two such active hydrogens, as shown in equation 3. Double Mannich reactions involving two active hydrogen atoms on the same carbon atom are well known. The diamides, including urea, could act as crosslinking agents with formaldehyde by reactions similar to those postulated by Fraenkel-Conrat and H. S. Olcott (3) and shown in equations 4 and 5. Perhaps the most interesting compound of this list is acetylacetone (II) which has the largest number of "active" hydrogen atoms.

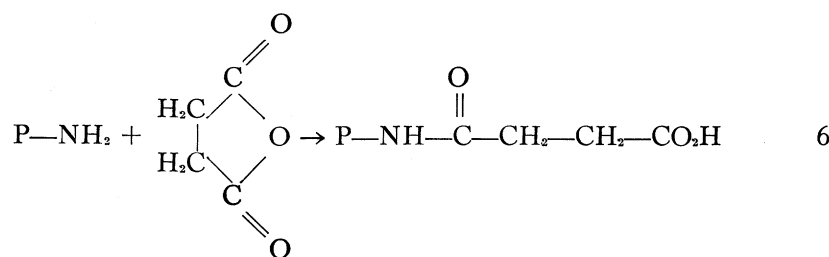


$$n = 0, 1 \text{ or } 2$$

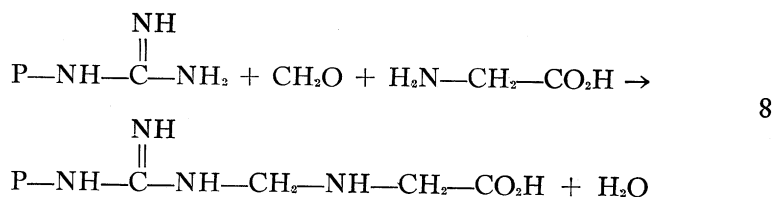
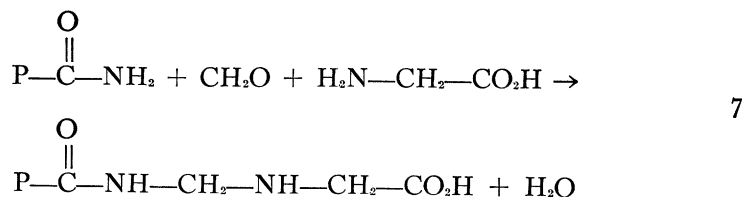
These equations show crosslinking or multipoint fixation. It is also probable that, because of stereochemical factors, unipoint fixation occurs simultaneously. A similar situation prevails in formaldehyde tanning. In contrast to the bonds formed by formaldehyde alone, however, bonds established through a Mannich reaction are extremely stable, even to acid hydrolysis. This is true for both unipoint and multipoint fixation.

These same compounds could, on reaction with formaldehyde and the protein, serve to introduce new or additional complexing sites for metals in subsequent mineral tannages. Since the carboxyl groups of collagen have been implicated (10) in the binding of chromium ions, the attachment of additional carboxyl groups by the reaction of the protein with formaldehyde and malonic acid is perhaps the most attractive of the possibilities. Other methods of attaching additional carboxyl groups have been investigated. Gustavson (11) has done this by treatment of hide powder or skins with succinic anhydride. The reaction in-

volved is shown in equation 6. This reaction had an additional effect; i.e., the basic amino groups were converted to neutral amido groups; and this, as shown



by Gustavson (11), had a detrimental effect on subsequent chrome tanning unless special conditions were used. Another approach to the introduction of carboxyl groups was that of Bowes and Elliot (12). They treated pieces of sheepskins with formaldehyde and glycine, among other amino acids. A reaction between these two reagents and the protein amido and guanidino groups would result in the introduction of new secondary amino groups as well as additional carboxyl groups. The reactions, as indicated by Bowes and Elliot (12), are shown in equations 7 and 8. This treatment proved to be effective in increasing the affinity for chromium salts and the hydrothermal stability of the chrome-tanned final product.



In the present work, small pieces of pickled cabretta skins were used as the source of collagen and were treated with formaldehyde and the active hydrogen compounds listed above. Four different sets of conditions (see experimental section) were tested for each compound. These were chosen from conditions under which reactions similar to the desired ones were known to take place. For convenience, these will be referred to by the approximate pH at which the treatment was performed. These were pH 2, 4, 8 and 10.

To test the potential of the Mannich reaction involving the above active hydrogen compounds as a tannage in itself, the shrinkage temperatures of the treated skins were determined (7) immediately after tanning, and the skins were then allowed to dry with staking. In each case, a control was run under the same conditions using formaldehyde alone. These results are summarized in Table I. Although it could be demonstrated by various means that a reaction had taken place between the collagen, formaldehyde, and the "active" hydrogen-atom-containing compounds, in only a few cases is there any improvement in the shrinkage temperature over that of the corresponding control. At pH 4, acetone, nitromethane, oxamide, and succinic diamide increased the  $T_s$  by about four or five degrees above that of the corresponding control. It is questionable whether any significance can be ascribed to these slight increases in  $T_s$ . The highest increase (5°C.) was obtained in the case of the succinic diamide. This test was repeated on a full skin which was then processed into finished garment leather with a regular pack in a tannery. The finished leather did not appear to have any advantages over the formaldehyde leather. In fact, the treatment proved to be unstable on aging. The  $T_s$  fell from 78°C. to 59°C. in nine months.

The treatments at pH 10 gave, in all cases including the control, a product which was full, soft, and in most cases fairly strong. This is in agreement with results found by Seligsberger and Sadlier (8) for formaldehyde alone. However, as far as the present investigation was concerned, in no case was there any improvement over the control sample which was tanned with formaldehyde alone.

TABLE I  
T<sub>s</sub> OF PRODUCTS FROM VARIOUS TREATMENTS

	pH 2 °C.	pH 4 °C.	pH 8 °C.	pH 10 °C.
Control††	78	77	83	85
Acetone	78	81	84	85
Acetylacetone	47*	76†	83†	83†
Dimethyl Malonate	76	77	83	82
Malonic Acid	69	79	83	82
Methyl Acetoacetate	47*	75†	83†	82†
Nitromethane	78	81	83	82**
Urea	65	76	83	83
Oxamide	79	81	84	81
Malonamide	67	80	83	81
Succinic Diamide	77	82‡	84	85

††Formaldehyde tannage.

\*Highly discolored, dried like parchment.

†Yellow.

\*\*Brown.

‡White, full, soft, very strong.

TABLE II  
CHROME RETANNAGE OF MANNICH PRODUCTS

	pH of Pretreatment							
	2		4		8		10	
	T <sub>s</sub> °C.	C <sub>12</sub> O <sub>3</sub> %	T <sub>s</sub> °C.	C <sub>12</sub> O <sub>3</sub> %	T <sub>s</sub> °C.	C <sub>12</sub> O <sub>3</sub> %	T <sub>s</sub> °C.	C <sub>12</sub> O <sub>3</sub> %
Control**	95	4.23	97	3.20	103	3.78	97	2.76
Acetone	95	3.74	94	2.46	104	3.87	100	2.69
Acetylacetone	89*	3.79	93*	2.62	97*	2.88	90*	1.37
Dimethyl Malonate	96	4.16	93	2.13	102	4.10	93	2.40
Malonic Acid	94	2.79	107	3.67	105	3.84	100	1.95
Methyl Acetoacetate	91*	4.16	91*	2.42	104	3.55	98	2.30
Nitromethane	95	3.68	95	2.42	104*	3.67	99†	2.42
Urea	100†	3.35	95	2.84	102	3.52	99	2.41
Oxamide	96	3.74	94	2.34	103	3.41	105	4.00
Malonamide	95	4.01	94	2.69	100	3.42	100	2.67
Succinic Diamide	96	3.94	94	2.56	103	3.91	103	2.63

**\*\*Formaldehyde tannage.**

\*These samples were all green and weak.

†Brown.;

† All  $T_s$  measurements of 100° or better were determined on freely suspended samples in a pressure apparatus (7).

These aliphatic "active" hydrogen-atom-containing compounds are in marked contrast to resorcinol and melamine, which also contain active hydrogen atoms but are of aromatic character. These latter two active compounds gave  $T_s$  values above 100°C. when used in the Mannich reaction with collagen, as reported by Windus (4, 5).

The Mannich reaction was also evaluated from the standpoint of binding capacity of the treated products for mineral tanning agents, such as chrome. For this purpose, the Mannich products were retanned with chrome and the  $T_s$  and chrome content evaluated (see Table II). It can readily be seen that there is no apparent correlation between the shrinkage temperatures and the  $Cr_2O_3$  contents. This is true not only for a given compound at the different pH's but also the different compounds at a given pH. We believe that this is due to the fact that we are not only influencing the ability of the protein to bind more or less chromium but also the extent of crosslinking by the chromium which is bound.

When  $T_s$  and  $Cr_2O_3$  content were used as criteria of fixation, only two treatments; namely, malonic acid at pH 4 and oxamide at pH 10, were notable (Table II). The former system gave values of 107°C. for the shrinkage temperature and resulted in a chrome uptake corresponding to 3.67 percent of  $Cr_2O_3$ . The latter system gave values of 105° for  $T_s$  and 4.00 percent for  $Cr_2O_3$  content.

From this preliminary survey, malonic acid and oxamide appeared as the most promising active hydrogen compounds in the Mannich reaction as applied to hide substance. Further tests on a scale of a full cabretta skin per test were conducted with these two active hydrogen compounds, and the Mannich pretreated skins were retanned with chrome. They were then processed into finished leather with a regular pack at a garment leather tannery. With the oxamide pretreatment, a poor leather resulted as judged visually. The malonic acid pretreatment, however, gave a leather that was judged to be of high quality. These encouraging results with malonic acid have stimulated further research on its reaction with formaldehyde and hide substance, and the results of this research are discussed in the following paper.

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